Dissecting the Intersection Between the *Rb/E2f* and *Myc* Pathways *in vivo*.

Honors Research Thesis

Presented in partial fulfillment of the requirements for graduation with honors research distinction in Molecular Genetics in the College of Natural and Mathematical Sciences at the Ohio State University

by

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ABSTRACT

Cancer can be defined as unregulated cell growth. This uncontrolled proliferation often stems from the disruption of transcriptional pathways. Some cell cycle regulators have been well characterized, however the interaction between some important pathways have not been thoroughly studied *in vivo*. The relationship between the important tumor suppressor Retinoblastoma (*Rb*) and the *E2F* family has been studied extensively. At the same time, *c-Myc* is a transcriptional activator that has been shown to be critical for proper proliferation, differentiation, and apoptosis. While the *Rb/E2F* and *c-Myc* pathways are well defined individually, their interaction *in vivo* is unknown. In this study, we first demonstrate that deletion of either *E2f1-3* or *c-Myc* are able to rescue an Rb deficient phenotype. Secondly, we show that combinatorial deletion of *E2f1-3* and *c-Myc* reveal a subset of genes that are synergistically regulated by both *E2f1-3* and *c-Myc*. These results suggest that the pathways of these transcription factors overlap more than originally thought, and further analysis of transcriptional affiliation will be paramount to understanding carcinogenesis.

INTRODUCTION

Retinoblastoma (*Rb*) is a tumor suppressor protein belonging to the pocket protein family, a group that also includes p107 and p130[1]. RB and the RB-like proteins are vital for normal cell division in mammals[2-3]. Most knowledge about RB's role in cell division centers on its interaction with the E2F family of proteins. There are currently eight known members of the *E2f* family[4-9]. *E2f1, E2f2*, and *E2f3* are classified as activators, while *E2f4-8* are classified as repressors (Figure 1)[4]. *E2f* activity is highly tissue and pathway dependent. For example, deletion of *E2F1* and *E2F2* can cause diabetes in mice[10].

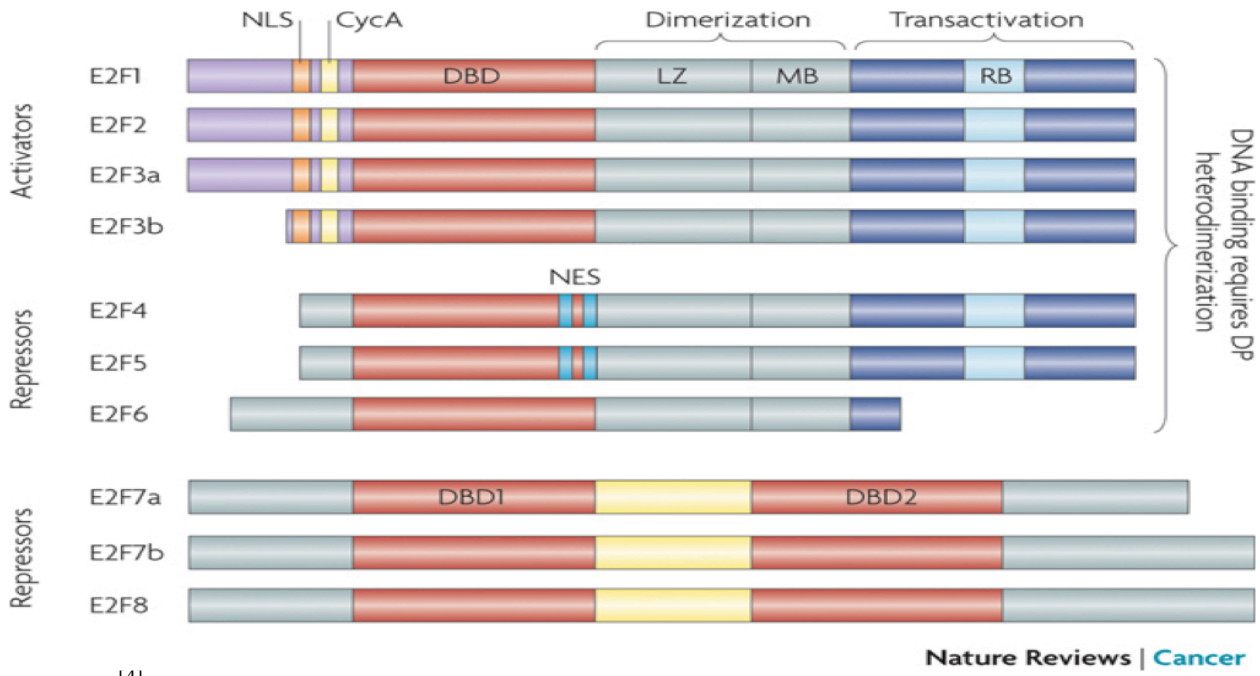

Figure 1[4]. *E2F* family diagram. Map of conserved or unique binding domains, dimerization domains, nuclear localization and export signals, as well as DNA binding domains across family members.

RB antagonizes E2F1-3 by physically sequestering them in the nucleus[11-13]. RB's ability to repress E2F1-3 activity is disrupted when it is phosphorylated by G1 regulated

cyclin dependent kinase (CDK) complexes[14-19]. Phosphorylation causes RB to release transcription factors E2F1-3, which then helps transition a cell into the DNA replication phase via transcription of their respective targets (Figure 2)[21]. Mutation or deletion of *Rb* allows this process to occur without check, and can ultimately lead to cancer[20]. RB deficiency has been identified in many types of cancer, adding to its importance in the search for cancer detection and treatment.

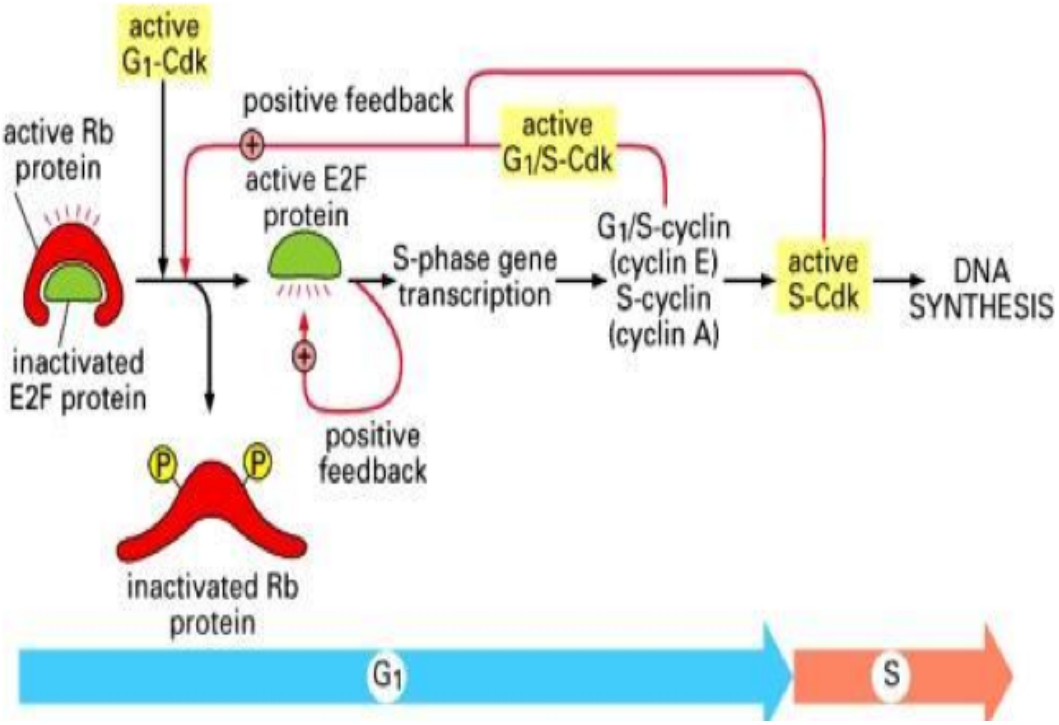

Figure 2[21]. Rb/E2f pathway. Rb represses E2f1-3 activity until it is phosphorylated by G1-CDK complexes. Once released, E2f1-3 push a cell into S phase by promoting transcription of S-cyclins and positive feedback for it's own transcription.

The *Myc* family of transcription factors (*c-Myc, N-Myc, L-Myc, S-Myc and B-Myc*) has been observed to mediate multiple biological processes including cell growth,

proliferation, differentiation and apoptosis[23-26]. *c-Myc*, a proto-oncogene known to be over expressed in cancer situations, has been shown to control the activation of the CDK complexes. The important Wnt, Shh and EGF pathways are known activators of *c-Myc*, another example of *c-Myc*'s importance in cancer pathways. *c-Myc* deletion has also shown promise as a tissue specific form of cancer therapy. *c-Myc* is a downstream player of the adenomatous polyposis coli (APC) tumor suppressor protein. Loss of APC leads to the uncontrolled proliferation of epithelial intestinal cells. When *c-Myc* was deleted in APC deficient cells, normal cell proliferation was restored[27-28]. Interestingly, c-MYC has also been recently recognized to function cooperatively with transcription factors E2F1-3 on processes such as cell proliferation and apoptosis[29-33]. There appears to be two canonical pathways that regulate cell proliferation, the *Apc/Myc* and the *Rb/E2F* pathways

In vitro-derived data shows that the c-MYC/RB gene products may functionally antagonize one another in the control of cell proliferation. However, there is no clear data indicating the physiological relevance data explaining the connection. Recent work by the Bremner and Leone groups shows that activation of *Rb* and downregulation of *c-Myc* expression are involved in regulating the cell's exit from the division cycle. In order to observe the effects of these proteins on the cell cycle, this study examines epithelial cells of the murine gastrointestinal system. This organ has a distinct and well-organized crypt-villus architecture that facilitates the study of proliferation and has a high cell turnover rate even in the adult mouse (Figure 3), important because some cell types such as nervous tissues stop dividing after a certain age[34]. The integrity of this structure is maintained by tight-controlled cell proliferation, differentiation and apoptosis.

It is clear that the *Rb/E2f* and *c-Myc* pathways are important for cell cycle control, and changes in their activity have been documented in many types of cancer. There have also been studies that suggest cooperation between the two pathways, yet they are not well characterized *in vivo* and the molecular mechanism is not well understood. Given the importance of these two pathways in carcinogenesis and suggestive evidence of their overlapping pathways, we have attempted to more concretely define the coordination between these two pathways *in vivo*.

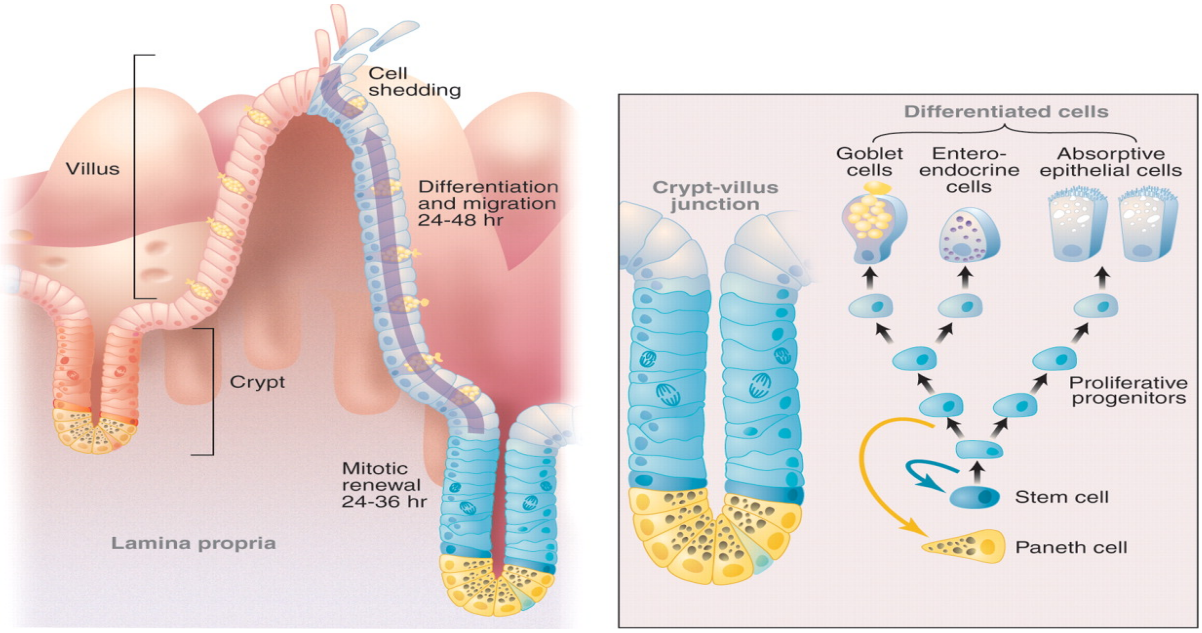

Figure 3[34]. Diagram of the small intestine crypt and villi structures. Stem cells in the crypts provide the progenitor cells that will develop into the differentiated villi epithelium. These cells migrate up the villi until they are shed at the top. The entire migration takes approximately 48-84 hours.

MATERIALS AND METHODS

Mouse Strains and Genotyping

All usage of mice has been approved by Institutional Animal Care and Use Committee at the Ohio State University. Ah-cre transgenic mice were bred with mice containing floxed genes of interest to obtain desired genotypes. Genotypes used in this study (*Ah-cre,* $Rb^{loxP/loxP}$, $E2f1^{-/-}$, $E2f2^{-/-}$, $E2f3^{loxP/loxP}$, *and* $Myc^{loxP/loxP}$) were tested using DNA isolated from tail samples and amplified using standard PCR. Primers used for this study include:

Ah-cre:
Ah1-fwd CCT GAC TAG CAT GGC GAT AC,
AhCre-2 ATT GCC CCT GTT TCA CTA TC.

Rb:
Rb18 GGC GTG TGC CAT CAA TG (WT)
Rb19e CTC AAG AGC TCA GAC TCA TGG (WT)
Rb212 GAA AGG AAA GTC AGG GAC ATT GGG (KO).

E2f1:
E2F1F AGC CAC TGG ATA TGA TTC TTG GAC (WT),
E2F1R AGA AGT CAC GCT ATG AAA CCT CAC (WT),
Neo AGT GCC AGC GGG GCT GCT AAA (KO).

c-Myc:
Myc 2 : AAT TTA AGC CTG ACC CCC GCG GCA (KO),
3'flox : TAC AGT CCC AAA GCC CCA GCC AAG (WT),
5'flox : CAC CGC CTA CAT CCT GTC CAT (WT).

Cre recombinase was stimulated via intraperitoneal injection of β-naphthoflavone at 2 months with 5 injections over 30 hours. The small intestine was harvested 2-5 days after the final injection, and either villi/crypt isolation performed or tissue fixation in formalin. BrdU was injected intrperitoneally injected two hours prior to dissection.

RNA

RNA was purified from either intestinal epithelial or crypt cells using Trizol and cleaned with Qiagen RNA miniprep columns. Microarray analysis was done by The Ohio State University Shared Resources Center using Affymetrix Mouse 30.2 chips.

qRT-PCR

cDNA was synthesized using RNA from isolated intestinal epithelium or crypt tissue and Invitrogen Supercript III Reverse Transcriptase Kit. qRT-PCR was performed using BIO-RAD iCycler with SYBR detection system. All samples were analyzed in triplicate and flourecense measured relative to GAPDH.

Staining

IHC and IF staining-Antibodies used for tissue staining: BrdU (Dako M0744), Phosphorylated Histone-3 (Millipore 06-570), Cleaved Caspase-3 (Cell Signaling, 9661L), Phosphorylated H2AX (Cell Signaling 9718P), Cyclin A2 (Santa Cruz sc-596), CDK1 (Santa Cruz sc-54), Mcm3 (Santa Cruz sc-9850), Pcna (Santa Cruz sc-56). Tissues used for staining were harvested and immediately fixed in formalin. Slide preparation was done by the Leone Histology Lab.

RESULTS

Loss of Either *E2f1-3* or *c-Myc* Rescues *Rb*-deficiency

To test whether *E2f1-3* and *c-Myc* (herein noted as *Myc*) are performing similar functions in regulation of the cell cycle, we first tested if ablation *c-Myc* can rescue an *Rb*-deficient cell similar to ablation of *E2f1-3*. *Ah-cre* transgenic mice were used for conditional knockout of genes of interest. To stimulate Cre recombinase, β-naphthoflavone was injected intraperitoneally and tissues harvested 2-5 days later. Deletion of *Rb* causes ectopic proliferation in the intestinal epithelium, without altering normal crypt division as observed using immunofluroescence detection of cell cycle markers BrdU and Phosphorylated Histone-3 (P-H3), (Fig. 4A,B). When E2f1-3 is deleted in Rb-deficient tissues, the ectopic proliferation in the epithelium is reduced, consistent with the current understanding of *E2f1-3* functioning as downstream effectors of *Rb*. Interestingly, deletion of *Myc* also alleviated the ectopic proliferation caused by deletion of Rb (Fig. 4A,B).

We then compared global gene expression to understand the basis of this genetic rescue. Groups used were: β-NF treated control (*Ctrl*), *Ah-cre* $Rb^{loxP/loxP}$ (*RbKO*), *Ah-cre* $Rb^{loxP/loxP}$ $E2f1^{-/-}$ $E2f2^{-/-}E2f3^{loxP/loxP}$ (*Rb/E2f QKO*) and *Ah-cre* $Rb^{loxP/loxP}$ $Myc^{loxP/loxP}$ (*Rb/Myc DKO*). Global gene expression levels were similar in the intestinal crypts between the four genetic groups. In the epithelial cells, *Rb* deletion corresponds to dramatic gene expression changes. Many of the genes affected by *Rb* deletion were rescued by additional deletion of either *E2f1-3* or *Myc* (data not shown). There was significant overlap between genes rescued by *E2f1-3* deletion and genes rescued by *Myc* deletion. Expression levels of these genes were validated using quantitative real-time PCR (qRT-PCR) (Fig. 4C).

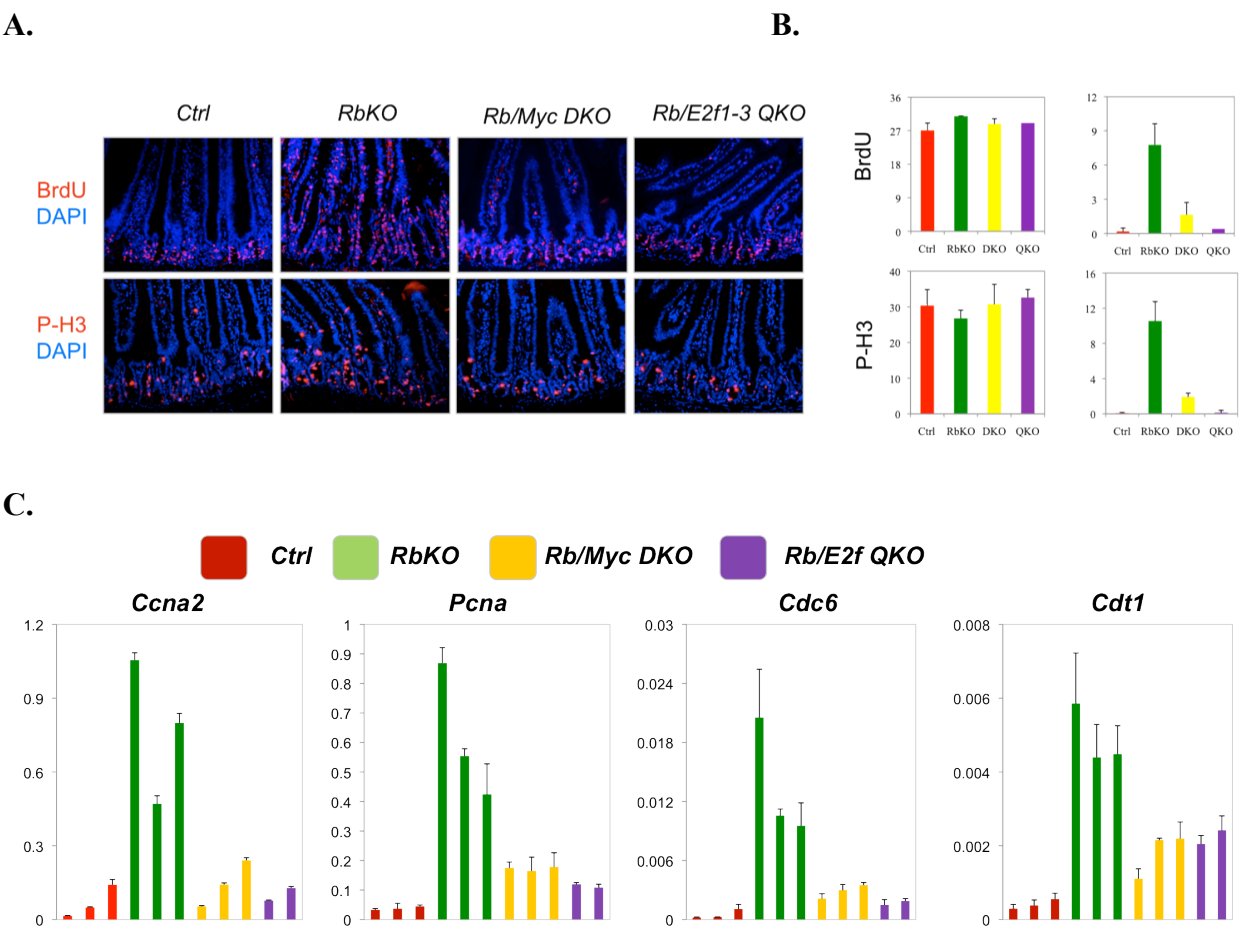

Figure 4. Genetic rescue of Rb-deficiency. A. Control, RbKO, Rb/Myc DKO, and Rb/E2f1-3 QKO intestinal slides were immunostained for BrdU(S-phase indicator) and P-H3 (mitosis indicator). DAPI was used as a nuclear counter stain. B. Quantification of percent positive BrdU or P-H3 cells in either intestinal crypt or villi sections (n=4). C. Expression levels of G1/S genes via cDNA synthesized from RNA of purified intestinal villi cells, relative to GAPDH.

Simultaneous Deletion of *E2f1-3* and *Myc* Results in Loss of Intestinal Homeostasis

The rescue of an *Rb*-deficient phenotype by deletion of both *E2f1-3* and *Myc* prompted us to investigate if there was any synergy between *E2f1-3* and *Myc*. To analyze if *E2f1-3* and *Myc* coordinate cell cycle *in vivo*, we studied the affects of simultaneous deletion of both *E2f1-3* and *Myc* on the small intestine epithelium. While *Ah-cre* $E2f1^{-/-}$ $E2f2^{-/-}$ $E2f3^{loxP/loxP}$ (*E2f TKO*) or *Ah-cre* $Myc^{loxP/loxP}$ (*MycKO*) intestines looked similar to β-NF treated control animals (*Ctrl*), simultaneous deletion of *E2f1-3* and *Myc* in *Ah-cre* $E2f1^{-/-}$ $E2f2^{-/-}$ $E2f3^{loxP/loxP}$ $Myc^{loxP/loxP}$ (*E2f/Myc QKO*) tissues were marked with complete loss of intestinal homeostasis (Fig. 5A). The disruption of the villi originates in the crypts because intact villi were observed in mice harvested one day after β-NF injection, while crypts progressively declined (Fig. 5E).

In order to examine if disruption of the cell cycle was contributing to the declining number of crypt cells in *E2f1-3/Myc* QKO mice, immunofluorescence staining of S phase indicator BrdU and mitosis indicator P-H3 was performed (Fig. 5B,C). DNA synthesis was unaffected in *E2f1-3*/*Myc* QKO crypts, but there was a drastic decline in the number of mitotic cells. These results suggest that simultaneous deletion of *E2f1-3* and *Myc* causes disruption of the G2/M transition, but not G1/S. Upon higher magnification, *E2f1-3/Myc* QKO crypt cells appeared to be larger and the chromatin less dense than Ctrl crypts (Fig 5D).

To assess if other cellular processes were affected by *E2f1-3/Myc* deletion, immunohistochemistry staining of cleaved caspase-3 and phosphorylated-H2AX was done to examine apoptosis and double-strand DNA breaks, respectively. Only *E2f1-3* TKO crypts showed elevated apoptosis, whereas both *Myc* KO and *E2f1-3/Myc* QKO crypts

showed extremely low levels of apoptosis (Fig. 6). Upon examination of *E2f1-3/Myc* QKO crypts at earlier time points, apoptotic events were still rare, indicating that additional deletion of Myc did not cause apoptotic cells to be removed earlier. However, both *E2f1-3* TKO and *E2f1-3/Myc* QKO crypts stained positive for double-strand DNA breaks (Fig. 6). This data suggests that deletion of *E2f1-3* is enough to initiate DNA damage and initiate the apoptosis program. However, *E2f1-3/Myc* QKO crypts could not successfully carryout apoptosis because the simultaneous deletion of *E2f1-3* with *Myc* caused arrest at mitosis and the corresponding checkpoint responses to DNA damage. It appears then that *E2f1-3* and *Myc* are not mandatory for proper G1/S phase transition in progenitor cells. However, they both are vital for proper G2/M phase transition.

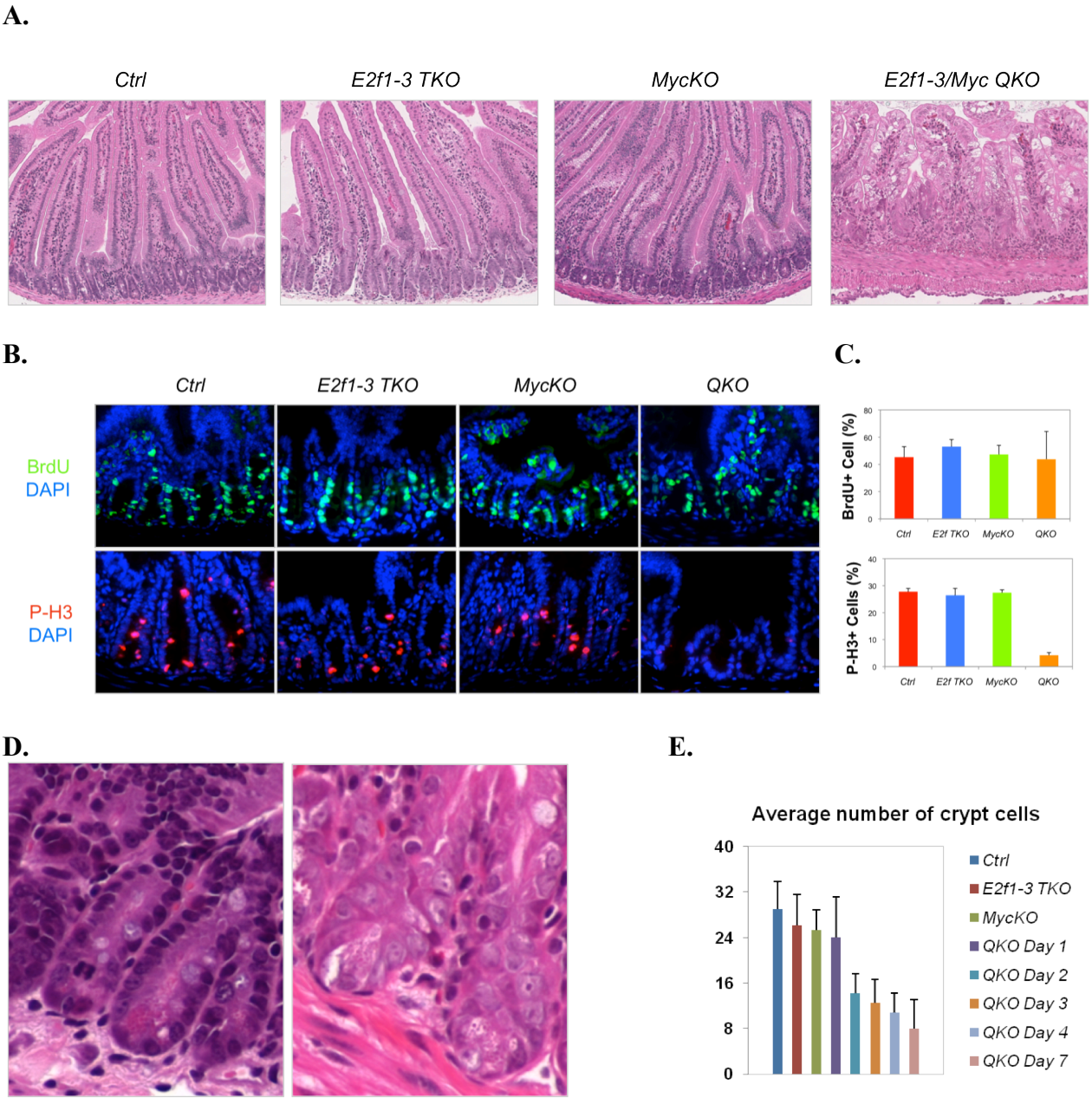

Figure 5. Loss of epithelial homeostasis in *E2f1-3/Myc* QKO mice. A. H&E stain of Control, *E2f1-3* TKO, *MycKO*, and *E2f1-3/Myc* QKO intestine. B. BrdU and P-H3 immunostained slides of intestinal crypt sections. DAPI used as a nuclear counter stain. C. Quantification of perfect positive BrdU or P-H3 cells in crypt sections (n=3). D. High magnification H&E stain of Control and QKO crypts, respectively. E. Quantification of average number of crypt cells per crypt in relation to genotype and time after injection of β-Naphthoflavone (n=2).

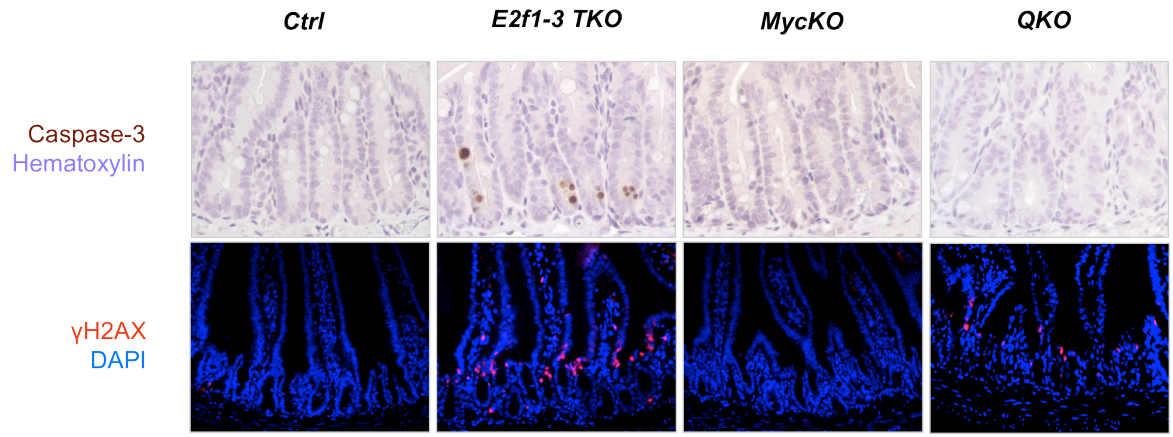

Figure 6. Mitotic arrest and DNA damage in *E2f1-3/Myc* QKO mice.
Immunofluorescence staining of Control, *E2f1-3* TKO, *MycKO*, and *E2f1-3/Myc* QKO intestinal crypt sections. DAPI used as a nuclear counter stain.

Combinatorial Transcriptional Regulation by *E2f1-3* and *Myc*

We have demonstrated that *E2f1-3* and *Myc* cooperate on certain aspects of the cell cycle. In order to understand pathway *E2f1-3* and *Myc* follow to facilitate this cooperation, we performed a microarray to assess the global gene expression of β-NF treated control, *E2f TKO*, *MycKO* and *E2f/Myc QKO* intestinal crypt cells. There were four areas of genes which resulted in changes of gene expression levels (Fig. 7A): The first category are genes which saw changes in gene expression in *E2f1-3* TKO, *Myc* KO, and *E2f1-3/Myc* QKO crypts, indicating they are regulated by both *Myc* and *E2f1-3*. The second and third group would be genes that were changed with either *E2f1-3* or *E2f1-3/Myc* deletion, and *Myc* or *E2f1-3/Myc* deletion. These two groups would be said to be regulated by *E2f1-3* or *Myc*, respectively. The fourth group of genes would be the category where gene expression only changed with the simultaneous deletion of *E2f1-3* and *Myc.* It is important to note that there is the possibility of genes being slightly misregulated in either the *E2f1-3* TKO or *Myc* KO and greatly misregulated in the *E2f1-3/Myc* QKO category, however only the statistically significant change in expression in the *E2f1-3/Myc* category was considered to be *E2f1-3/Myc* synergistic. In order to validate the gene expression changes found in the microarray, mRNA isolated from β-NF treated control, *E2f TKO*, *MycKO* and *E2f/Myc QKO* intestinal crypt cells was used for reverse transcription and quantitative real-time PCR for a group of genes found in the fourth microarray category (Fig. 7C). There was a subset of G2/M genes (*Ccnb1, Cdc2, Cdc20*) that showed no changed in gene expression in the *E2f1-3* TKO or *Myc* KO cells, and were down-regulated in the *E2f1-3/Myc* QKO cells. Important G1/S transition genes (*Pcna, Mcm3, Cdc6*) were found to exhibit similar gene expression levels in all four genotypes.

Immunofluorescence for the G2/M subset of genes was done to show protein levels in the *Ctrl*, *E2f1-3* TKO, *Myc* KO, and *E2f1-3/Myc* QKO crypts (Fig. 7B). The staining results recapitulate the results from the microarray and qRT-PCR, there is a unique subset of G2/M transition proteins that are misregulated with combinatorial deletion of *E2f1-3* and *Myc*, resulting in a decrease of both mRNA and functional protein. This data suggests that the G1/S transition is not affected by simultaneous deletion of *E2f1-3* and *Myc*, other than the incurred DNA damage. However, the G2/M transition relies on cooperation from both *E2f1-3* and *Myc*.

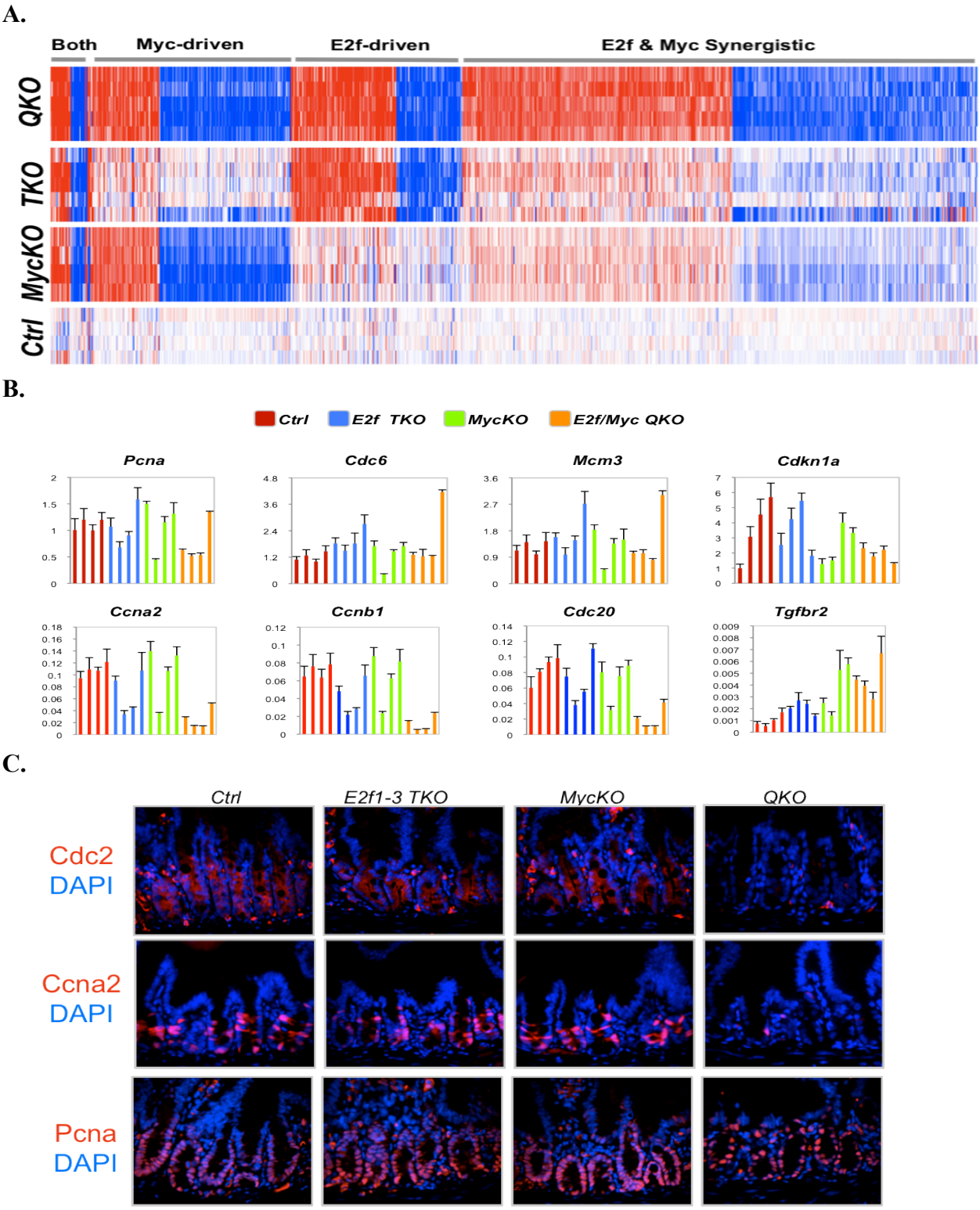

Figure 7. Combinatorial regulation of transcriptional programs by *E2f1-3* and *Myc*. A. Microarray results from cDNA synthesized from intestinal crypts ($p<0.05$). B. Expression levels via cDNA from purified intestinal crypt cells, relative to GAPDH. C. Immunofluorescence staining of intestinal crypts. DAPI used as a nuclear counter stain.

DISCUSSION

This study aimed to investigate the functional interaction between two well-characterized transcriptional pathways in the context of cell cycle control. The *Rb/E2f* pathway has long been known to be essential for proper G1/S phase transition, and the *E2f* family has been shown to be important activators and repressors of cellular activities. The *c-Myc* oncogene has also been shown to be a vital activator of cellular growth, differentiation, and apoptosis. *Rb, E2f*, and *c-Myc* have all been identified to be misregulated in many types of cancers, adding to their importance in regulation of the cell cycle. Despite the vast knowledge of these proteins, the understanding of their cooperation is lacking.

In this paper, we have shown that *Myc* and *E2f1-3* are essential for proper cell cycle control and intestinal cpithelium homeostasis. From this, we demonstrate that *E2f1-3* and *Myc* have distinct, yet overlapping transcriptional networks they regulate. A key area of synergy identified for *E2f1-3* and *Myc* is the G2/M phase transition in intestinal crypt cells. Simultaneous deletion of *E2f1-3* and *Myc* results in DNA damage, arrest of mitosis, and failure of apoptosis, which results in atrophic intestinal villi.

In an *Rb*-deficient context, deletion of either *E2f1-3* or *Myc* is sufficient to rescue ectopic cellular proliferation in the intestinal villi. Because the rescued epithelium exhibits close to normal DNA synthesis and cell division, *E2f1-3* and *Myc* are important in activating both G1/S and G2/M phase transitions. This suggests that in a cancer context, or loss of a tumor supressor, both *E2f1-3* and *Myc* are activating ectopic cell proliferation. In both cases, the *E2f1-3* and *Myc* transcriptional networks clearly overlap more than originally thought. This knowledge is extremely important in the fight to cure cancer

because cancer is defined by misregulation of transcriptional pathways. Unless researchers are able to clearly connect transcriptional targets in the interlocking pathways already characterized, we will not ble able to fully understand the process of carcinogenesis, nor will researchers be able to create effective plans of early detection and treatment without all the dots on the cancer map connected.

While we have shown that *E2f1-3* and *Myc* cooperate to regulate certain cellular processes, the molecular mechanism by which they regulate the activation of those pathways is yet to be determined. To do this, chromosomal immunoprecipitation sequencing (ChIP-seq) could be used to identify the DNA binding loci of given transcription factors. By fixing DNA/protein complexes in the intestinal epithelium with formaldehyde, we will be able to pull down sequences bound directly by either *c-Myc* or the *E2f* activators. Once the complexes are isolated and the DNA fragments amplified and sequenced, we will be able to analyze the direct targets of *Myc* and *E2f1-3* in the intestinal epithelium. We hypothesize that a subset of the *E2f1-3/Myc* synergistic genes obtained by the microarray will be direct targest of both *E2f1-3* and *Myc*. This would suggest that *E2f1-3* and *Myc* co-regulate a subset of genes by direct binding to their DNA sequences. If this is the case, this knowledge would help define the interaction between two important trasncription factors *in vivo* both in a normal and *Rb*-deficient (cancer) context.

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